Insulin Activates Glycogen Synthase in Cultured Human Fibroblasts

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SUMMARY

To develop a technique for examining short term insulin effects in cultured human fibroblasts, the effect of insulin on glycogen synthase was determined. Fibroblasts contain detectable glycogen synthase activity which can be converted from the D form, dependent on glucose-6-phosphate for activity, to the I form, which is independent of glucose-6-phosphate, in the presence of insulin. In the basal state, about 6-20% of the glycogen synthase is independent activity. This increases to between 30 and 60 %I activity after insulin stimulation. Stimulation is seen with insulin concentrations as low as 10^{-9} M, although maximal stimulation requires 10^{-7} – 10^{-6} M insulin. The effect of insulin is rapid, reaching a maximum within 20 min of incubation. Incubating the cells in fresh media without serum and glucose for up to 24 h before assay enhances the cellular response to insulin. Glucose has only a small, transient effect on the conversion of the enzyme from the D to the I form in the absence of insulin. These data demonstrate that human fibroblasts possess insulin-sensitive glycogen synthase, which may be used as a marker of metabolic response in disease states.
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Cultured human fibroblasts have proved to be an extremely useful tool in the elucidation of the mechanism of a number of metabolic disorders.[1] The accessibility of these cells, their amenability to biochemical analysis, and their maintenance of a stable genetic complement make them ideal in studies of human disease in vitro. Human fibroblasts are also insulin responsive in culture.[2–4] In most cases, however, the effect of insulin is small and, frequently, poorly reproducible, limiting the usefulness of the human fibroblast in studies of diabetes and other disorders of carbohydrate metabolism.

In a now classic series of experiments by Larner and his co-workers, it was shown that, in both muscle and adipose tissue, insulin stimulates the conversion of the enzyme glycogen synthase (uridine diphosphate glucose: glycogen glucosyl-transferase (EC 2.4.1.11)) from a form dependent on glucose-6-phosphate for activity (the D form) to a form independent of glucose-6-phosphate (the I form).[5] Since that time, glycogen synthase activity has been described in other tissues and cultured cells, including human fibroblasts.[6] In the latter case, however, data concerning either insulin or glucose regulation of the enzyme has been limited.[7] In the present study we characterized the glycogen synthase activity in cultured human skin fibroblasts and attempted to determine the roles of insulin and glucose in regulation of the D and I forms. In addition, we investigated the effect of the duration of cells in culture and their previous nutritional status on both the basal activity and the insulin-sensitive independent enzyme activity.

MATERIALS

The following compounds were obtained from the indicated sources: Eagle's minimal essential media, glucose-free media,* and serum-free media from the NIH Media Unit; fetal bovine serum (FBS) from Flow Laboratories, Rockville, MD; rabbit liver glycogen, glucose-6-phosphate, and uridine diphosphoglucose from Sigma Chemical Co., St. Louis, MO; ^{14}C-UDPG, specific activity of 229 mCi/mmol, from New England Nuclear, Boston, MA; Gelman chromatography media from Arthur H. Thomas Co., Philadelphia, PA; MSA from Collaborative Research, Waltham, MA. Tissue culture flasks were obtained from Falcon Plastics. All other chemicals were of reagent grade and were obtained from standard chemical supply houses.

METHODS

Human fibroblast cultures were initiated from punch biopsies of the forearm from healthy normal volunteers (three

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* Abbreviations used: G-6-P: glucose-6-phosphate; D: dependent form of glycogen synthase; I: independent form of glycogen synthase; UDPG: uridine diphosphoglucose; SF: serum-free Eagle's minimal essential media; and GSF: glucose-free and serum-free Eagle's minimal essential media.

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women, aged 19-20, and one man, aged 40) and were grown in Eagle's minimal essential media supplemented with 20% FBS and nonessential amino acids in a humidified CO_2 incubator at 37°C. For routine passage, the cells were subcultured weekly at a 1:3 dilution in 75 cm^2 flasks. For experiments, 2×10^5 cells were incubated in 20 × 100 mm culture dishes and were used with or without subsequent feedings as indicated. Cells were used between the fourth and eighteenth passages and were routinely negative when tested for mycoplasma contamination (Microbiological Associates).

Glycogen synthase assay. For assaying for glycogen synthase, the growth media were replaced by 3 ml Eagle's minimal essential media without glucose, supplemented with 1 mg/ml bovine serum albumin (BSA) and additions as indicated. Cells were incubated for 30′ at 37°C in a humidified CO_2 incubator, after which time the media were aspirated and 2 ml cold NaF-EDTA solution (100 mM NaF and 10 mM EDTA) was added for 15-30 s. The cells were scraped into tubes using a rubber policeman, centrifuged, and the pellet resuspended in NaF-EDTA solution. The cells were then disrupted by sonication with a Biosonic III instrument at a setting of 30 for 5 s. The particulate fraction was removed by centrifugation for 2 min at 10,000 rpm in a Beckman model B centrifuge, and the supernatant was assayed for glycogen synthase activity.

The enzyme assay was conducted using a modification of the method of Thomas:[8] 30 μl of the cell supernatant was added to 60 μl of a solution containing 50 mM Tris-HCl (pH 7.8), 20 mM EDTA, 25 mM NaF, 10 mg/ml glycogen, 6.7 mM UDPG, approximately 100,000 cpm ^{14}C-UDPG, (sp act = 229 mCi/ml) with or without 7.2 mM G-6-P. Incubation was for 20′ at 30°C, after which time 50 μl of the reaction mixture was spotted on Gelman chromatographic strips, dried, and then subjected to ascending chromatography in 66% ethanol with 0.1M ammonium acetate according to the method of Huang and Cabib.[9] The strips were again air dried, cut, and counted in a Searle Delta 300 liquid scintillation system using ACS as the scintillation cocktail. Glycogen synthase I activity was defined as that measured in the absence of G-6-P, and total activity as that measured in the presence of G-6-P.[10] In our hands, this method yields results similar to those obtained with the method of Larner.[5] Proteins were determined by the method of Lowry et al.[11] using bovine serum albumin as a standard. Glycogens were determined by a modification of the method of Lo et al.[12] using rabbit liver glycogen as a standard.

RESULTS

Cultured human fibroblasts possess glycogen synthase activity capable of incorporating ^{14}C-UDP glucose into glycogen. When increasing amounts of cell supernatant protein were added to the glycogen synthase assay, there was a linear increase in the percent of ^{14}C-UDPG incorporated into glycogen as determined by chromatographic separation in ethanol-ammonium acetate buffer (Figure 1A). This assay was conducted in the presence of glucose-6-phosphate, and it represents total activity. In three experiments in which the protein concentration in the cell supernatants was measured on each sample in the assay, 0.39 ± 0.05 nmol of UDP ^{14}C-glucose was incorporated per milligram cell supernatant protein ($\bar{x}$ ± SD). However, both in the basal state

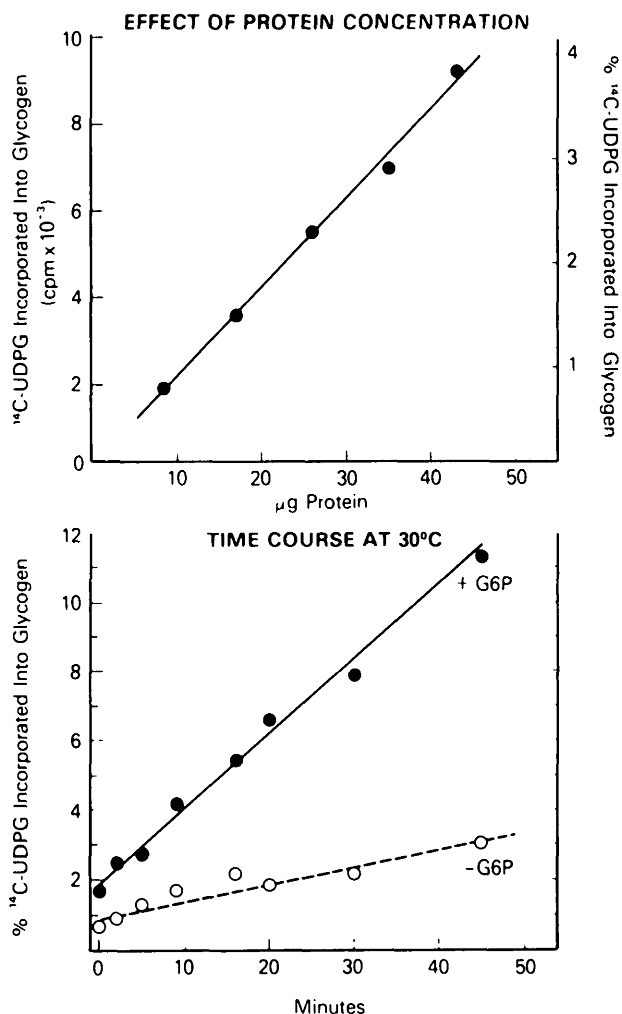

FIGURE 1. (Upper Panel) Effect of protein concentration on glycogen synthase activity. A pooled supernatant from insulin-stimulated and control cells was assayed in increasing volumes to achieve the indicated protein concentrations. The assay was carried out in the presence of G-6-P as described in MATERIALS AND METHODS. The data are expressed as both cpm of ^{14}C-UDPG incorporated into glycogen (left axis) and the percent of added label incorporated into glycogen (right axis). (Lower Panel) Time course at 30°C. A pooled supernatant from insulin-stimulated and control cells was assayed in the presence (●) or absence (○) of G-6-P as described in MATERIALS AND METHODS. Data are expressed as percent ^{14}C-UDPG incorporated into glycogen.

and after insulin stimulation, a fraction of the activity (10-30%) did not require glucose-6-phosphate for activity, i.e., it was present in the independent form. The incorporation of ^{14}C-UDP glucose into glycogen in both the presence and the absence of glucose-6-phosphate was linear for up to 45 min at 30°C (Figure 1, bottom). For all subsequent assays, a 20 min time period was used to ensure adequate activity while remaining on a linear portion of the curve.

The percent of G-6-P–independent glycogen synthase activity and its response to insulin varied with the duration of cells in culture (Figure 2). Early after plating (3 days), the percent of independent glycogen synthase activity was relatively high, and the stimulation by a maximal concentration of insulin ($1.6 \times 10^{-7} - 1.6 \times 10^{-6}$ M, 1 μg/ml = 1.6×10^{-7} M) was small. As the fibroblasts became confluent, the percent independent (I) basal activity decreased; that is, more of the glycogen synthase activity was in the D form. At the

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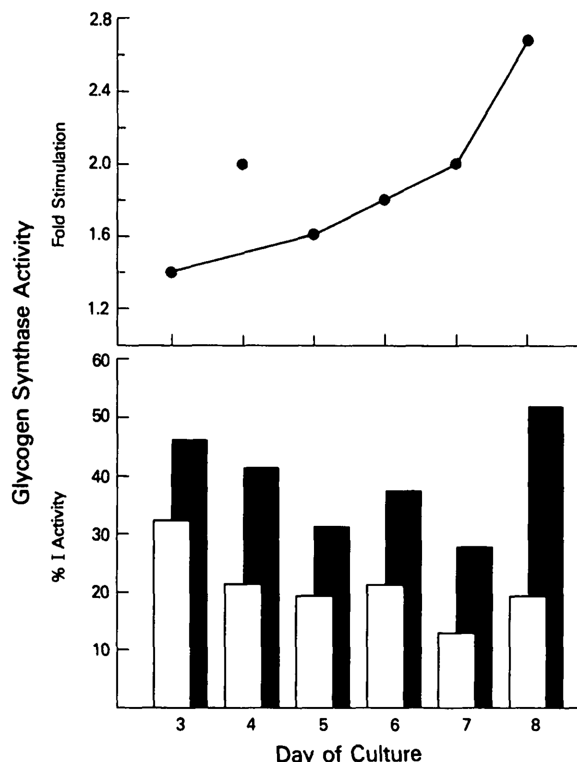

FIGURE 2. Glycogen synthase activity as a function of days in culture. Cells were plated on day 0 and then assayed without media change on the indicated days in the presence (solid bars) and absence (open bars) of 1.6 × 10⁻⁶ M insulin as described in MATERIALS AND METHODS. Data are expressed as %I activity in the lower panel and as the fold increase of insulin-stimulated cells over unstimulated cells in the upper panel.

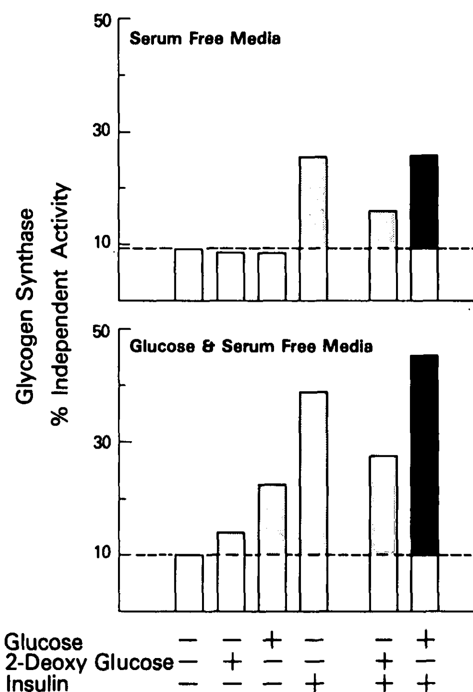

FIGURE 3. Effect of glucose deprivation on stimulation. Confluent 7-day-old cultures were changed to either SF media (upper panel) or to GSF media (lower panel) 24 h before assay. The cells were then incubated for 30 min in fresh GSF media with the following additions as indicated: D-glucose, 1 mg/ml; 2-deoxy-glucose, 1 mg/ml; insulin, 1.6 × 10⁻⁶ M. Data are expressed as %I activity as described in METHODS.

same time, the ability of insulin to stimulate the conversion of the D to the I form increased. After 8 days of culture, insulin stimulated the %I activity by 2.7 fold. No systematic change in total glycogen synthase activity was observed throughout the time frame studied. For all subsequent experiments, cells were used only 8-10 days after plating.

The finding that the cells became more insulin responsive after longer periods of culture suggested that depletion of nutrients from the media might increase insulin responsiveness. To investigate this in a more systematic way, cells were deprived of either serum or of serum and glucose for 24 h before study. As can be seen in Figure 3, depriving the cells of serum and glucose for 24 h before assay had little effect on the basal %I activity but enhanced the response of the cells to both insulin and to insulin with glucose. Typically, insulin increased the %I activity twofold to fourfold over unstimulated levels if the cells were kept in GSF media for 24 h before study.

The time course of the effect of glucose and serum deprivation is shown in more detail in Figure 4. Note that the absence of glucose and serum for up to 48 h has little effect on the percent independent activity in the basal state, but, after as little as 2 h, there was some increase in insulin-stimulated glycogen synthase %I activity. This increased progressively, reaching a maximum at 24 h of deprivation. The glycogen content of the cells after 24 h in GSF media did not change significantly. The starved cultures contained 217 ± 66 μg glycogen/mg protein ($\bar{x}$ ± SD, N = 12 experiments) as compared with unchanged cultures, which contained 239 ± 63 μg glycogen/mg protein.

As with adipose tissue,[5] the time course of insulin-stimulated conversion of glycogen synthase from the D to the I form was rapid (Figure 5). Whether or not the cells were preincubated for 24 h in glucose- and serum-free media, the glycogen synthase–independent activity was maximally stimulated by insulin within 20-30 min and remained stably elevated for up to 90 min. Half-maximal stimulation of glycogen synthase occurred within 5 to 15 min. Insulin, however, had no consistent effect on total glycogen synthase activity (data not shown). Also, there was no change in the basal %I activity of the enzyme during the 90 min period of incubation in media without insulin. The basal %I activity of fibroblasts under our culture and assay conditions was 12.8 ± 6.8 ($\bar{x}$ ± SD; N = 51 experiments on four control cell strains).

Previous studies in both adipose tissue and muscle[5,15] suggested an important role for glucose or glucose-6-phosphate in modulation of glycogen synthase activity. To explore this effect in the fibroblast, the effect of glucose was investigated over the range of 0-50 mg/ml. Although an occasional experiment showed a small effect of glucose (Figure 3) on the basal and stimulated %I activity following a 30′ incubation, more systematic studies failed to reveal any consistent or reproducible response to glucose in this time frame in either the presence or absence of insulin. In experiments using much shorter incubation times, i.e., 2-12 min, glucose did have a consistent, although transient, stimulatory effect (Figure 6). This effect was seen best at 4 h of pretreatment with GSF media, rather than the usual 24 h. Similarly, 2-deoxyglucose occasionally showed a small

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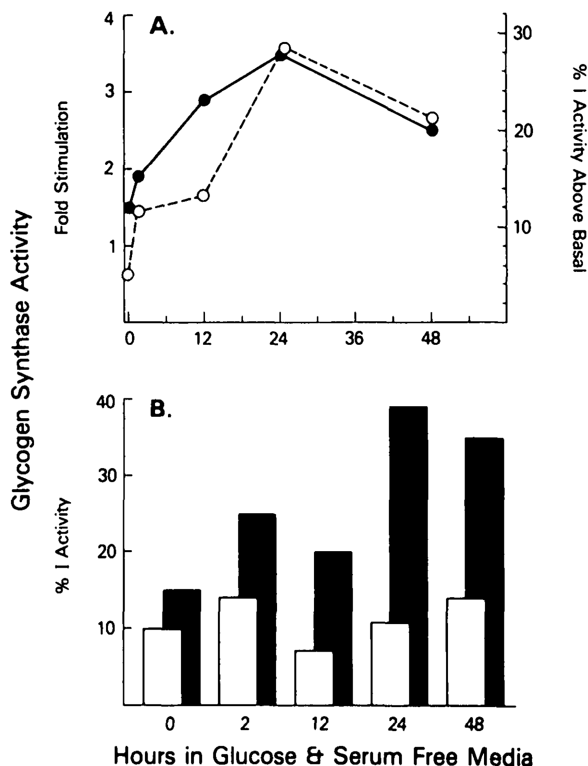


FIGURE 4. Time course of glucose and serum deprivation. Confluent cultures in sets of four dishes were changed to GSF media for the indicated times before being assayed for glycogen synthase activity. The change of media was made at appropriate times to allow for the assay of enzyme activity of all cells at the same time. Thirty minutes before harvesting the cells, insulin (1.6×10^{-6}M) was added to two dishes of each set. Glycogen synthase activity in the presence (closed bars) and absence (open bars) of insulin was assayed as described in METHODS. Data are expressed as %I activity in the lower panel. In the upper panel, the data are expressed as the ratio of the insulin-stimulated to the unstimulated activity on the left ordinate (●–●) and as the absolute increase in %I activity on the right ordinate (O---O).

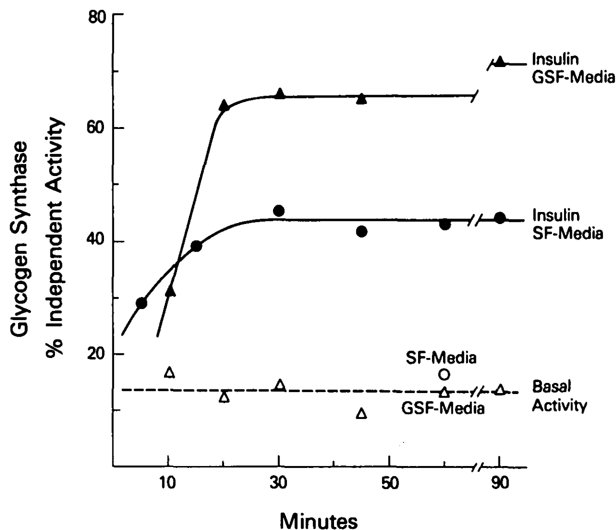


FIGURE 5. Time course of insulin stimulation. Confluent cultures were incubated for 24 h in either SF media (●,O) or GSF media (▲,△) before addition of 1.6×10^{-6} M insulin (solid symbols) or control media (open symbols). Additions of insulin and control media were made at appropriate times during the 90 min of incubation to give the indicated exposure times. Data are expressed as %I activity.

enhancement of basal or insulin-stimulated activity (Figure 3), but this was not reproducible under the usual assay conditions.

In contrast with the marked sensitivity of glycogen synthase to insulin in the adipose cell,[13] stimulation of glycogen synthase in the fibroblast requires relatively high concentrations of insulin. Reproducible stimulation was observed with an insulin concentration of 1.6×10^{-9} M, and a small effect of insulin was observed in some experiments at concentrations as low as 1.6×10^{-10} M. The effect of insulin was maximal at a concentration of 1.6×10^{-7} M, and half-maximal stimulation varied from $1.6 - 10 \times 10^{-9}$ M (i.e., 10-60 ng/ml) (Figure 7). The insulin dose-response curve was similar in the presence or absence of D-glucose (data not shown).

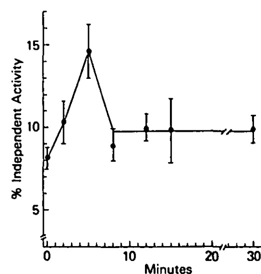
Fibroblasts possess receptors for both insulin and the insulin-like growth factors.[16,17] One such growth factor is multiplication-stimulating activity (MSA). MSA and insulin share an affinity for each other's receptors. MSA was tenfold less potent than insulin in stimulating %I activity (Table 1). This is more potent than such insulin-like effects as glucose oxidation in other tissues, where MSA is generally only about 1% as active as insulin.[18] In more recent experiments with different lots of MSA supplied by the same company, MSA was equipotent with insulin at the concentrations tested. Until standardized preparations of MSA are available, interpretation of these data is difficult. Antibody to the insulin receptor obtained from a patient with the type B syndrome of insulin resistance and acanthosis nigricans (patient B-2[18]) also was able to stimulate an increase in %I activity (Table 1). As with insulin, however, antibody stimulation of fibroblasts required somewhat higher concentrations than were needed to stimulate adipocytes.[20]

DISCUSSION

Glycogen synthase, the key enzyme in the regulation of glycogen synthesis, was previously demonstrated in a wide variety of cells,[10,21–23] including cultured human diploid fibroblasts.[6] The conversion of the D (or b) form, whose activity depends on the presence of glucose-6-phosphate acting as an allosteric activator, to the I (or a) form, which is independent of glucose-6-phosphate for activity, appears to involve dephosphorylation of the enzyme.[10] Although other insulin effects have been demonstrated in human fibroblasts, the ability of insulin to alter the glycogen synthase activity has not been previously reported, except in abstract form.[7]

In the present study, we demonstrated that glycogen syn-

FIGURE 6. Glucose time course. Confluent cells were changed to GSF media for 4 h and then incubated with 2 mg/ml D-glucose for the indicated times. Data are expressed as the average ± SEM for four experiments.



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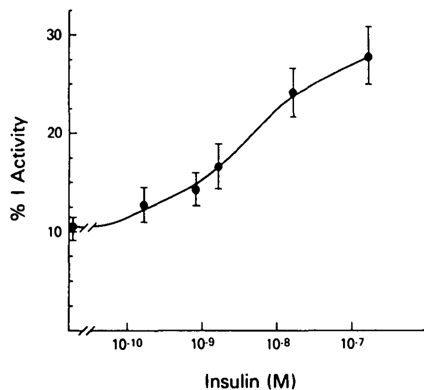

FIGURE 7. Insulin dose response. Confluent cells were changed to GSF media for 24 h and then incubated for 30 min in the indicated concentrations of insulin before being assayed for glycogen synthase activity. Data are expressed as %I activity ± SEM for a total of 12 experiments on four control subjects.

thase activity in human fibroblasts can be converted from the G-6-P–dependent (D) to the G-6-P–independent (I) form upon incubation with insulin. As seen with other insulin-responsive tissue, this increase in the percent I activity occurs with little or no change in total enzyme. The stimulation is rapid, and the cells typically display a twofold to fourfold increase in their percent I activity. This is similar in magnitude to the increase in activity observed in freshly isolated rat adipocytes.[13] In the adipocyte, however, glucose added to the incubation media during the stimulation period results in an increased percent I activity. This is thought to result from the formation of glucose-6-phosphate, since glucose analogues which can undergo phosphorylation mimic this effect, whereas those analogues which cannot be phosphorylated are without effect on glycogen synthase activity.[5,15] In this respect, the fibroblast behaves somewhat differently from the adipocyte, since only a slight, transient effect of glucose could be demonstrated on glycogen synthase activity in either the presence or absence of insulin. Similarly, 2-deoxyglucose was not consistently stimulatory. We cannot exclude some role for G-6-P in activation of the enzyme in cultured human fibroblasts, since we have not measured the concentration of this metabolite in the intracellular water.

Although it is generally agreed that activation of glycogen synthase involves dephosphorylation, the mechanism(s) by which insulin and glucose induce this change is complex and still uncertain. Lawrence et al. presented data to suggest that insulin exerts its effect in adipose tissue in two ways.[13] First, by activating glucose transport, with a resultant increase in glucose-6-phosphate which binds to glycogen synthase, making it a better substrate for the phosphatase, and secondly, by some less well defined effect of insulin independent of glucose transport. Nuttall et al.[24] presented data suggesting that the latter effect in muscle may be the result of activation of synthase phosphatase activity.

Insulin stimulation of glycogen synthase in the cultured fibroblast also differs from that in adipocytes in that higher concentrations of insulin (10^{-9} – 10^{-8} M) are required to produce the biologic effect. It is known that the human fibroblast has receptors for insulin-like growth factors which bind multiplication-stimulating activity (MSA) and insulin with almost equal potency.[16] Whether insulin activates glycogen

TABLE 1
Stimulation of %I activity in human fibroblasts

	Concentration		Stimulation (fold increase)
Experiment 1*	Insulin (nM)	MSA (nM)	
	16.7	—	2.6
	83.3	—	3.0
	1670.0	—	3.3
	—	11.5	1.45
	—	57.5	2.6
	—	1150.0	2.75
Experiment 2†	Insulin (nM)	Anti-R (μg/ml)	
	0.167	—	1.2
	1.67	—	2.2
	16.7	—	2.9
	—	20	2.65

* Basal of 11 %I activity
† Basal of 12.5 %I activity
Data are expressed as the fold increase above basal %I activity after 30′ of incubation with the indicated concentrations of insulin, MSA, or anti-insulin receptor antibody (Anti-R). (1 nM insulin = 6 ng/ml; 1 nM MSA = 8.7 ng/ml).

synthase in fibroblasts by binding to the insulin receptor, or to a growth factor receptor, or to both remains uncertain. Certainly, in the concentration range used, insulin will bind to both insulin and growth factor receptors.

Antibody to the insulin receptor also activates glycogen synthase, but it requires relatively high concentrations. These findings are consistent with activation of glycogen synthase by the binding of the stimulatory ligands to either the insulin or the insulin-like growth factor receptor. The concentrations of insulin used with fibroblasts are similar to the concentrations required for stimulation of glycogen synthase activity in HeLa cells[25] as well as to the dose response reported in abstract form by Craig and Larner[7] for fibroblasts.

One of the most interesting aspects of the present study is the marked change in glycogen synthase activity with both duration of culture and nutrient depletion. After the first several days of culture, there is a substantial decrease in the percent of independent glycogen synthase activity, while the %I activity after insulin stimulation increases. This can be further magnified by depriving the cells of all serum and glucose for 24 h before use in the assay. The reason for this marked effect of nutrient depletion is not clear. When adipose tissue is taken from fasting rodents, it has both a decreased glycogen content and a decreased ability to incorporate labeled glucose into glycogen.[26] Both these findings rapidly revert upon refeeding of the intact animal. Similarly, in cultured cells, there is often a decrease in glycogen stores with prolonged nutrient depletion. However, with HeLa cells, as well as two cell lines of neuronal origin, this was associated with an increase in the basal %I activity of glycogen synthase.[23,25] Huang found that glycogen synthase in choriocarcinoma cells is not able to be converted from the D to the I form, except after glucose starvation.[27] The effect in these cells depends on protein synthesis during the starvation period. He interprets his data as suggesting that glycogen synthase is partially dephosphorylated during starvation, making the D form of the enzyme a better

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substrate for phosphoprotein phosphatase. An increase in insulin-stimulated D-glucose transport[28-30] and in insulin stimulation of ^{3}H-thymidine incorporation into DNA[4] has been observed in fibroblasts after glucose or serum starvation, although in many cases this is due to differences in basal activity. In our hands, nutrient depletion results primarily in an increased insulin responsiveness without affecting basal enzyme activity or glycogen stores.

In summary, in the present study we demonstrated that human fibroblasts contain glycogen synthase activity, and there is a conversion from the dependent to the independent form in the presence of insulin. There appears to be little effect, however, of glucose on enzyme activity. The effects observed with glycogen synthase are reproducible and, compared with most other insulin effects in human fibroblasts, of a relatively large order of magnitude. This enzyme may therefore provide a useful marker for measurement of insulin action in human fibroblasts obtained from patients with diabetes and other disorders of carbohydrate metabolism.

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